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REVIEW ARTICLE

Protein kinase Cδ

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The protein kinase C (PKC) family consists of 11 isoenzymes that, due to structural and enzymatic differences, can be subdivided into three groups: The Ca^{2+}-dependent, diacylglycerol (DAG)-activated cPKCs (conventional PKCs: α, $β_1$, $β_2$, γ); the Ca^{2+}-independent, DAG-activated nPKCs (novel PKCs: δ, ε, η, θ, μ), and the Ca^{2+}-dependent, DAG non-responsive aPKCs (atypical PKCs: ζ, λ/ι). PKCμ is a novel PKC, but with some special structural and enzymatic properties.

Keywords: protein kinase Cδ; activation; autophosphorylation; down-regulation; structure; tyrosine phosphorylation; function.

Protein kinase Cδ (PKCδ) is the most thoroughly studied member of the nPKC subfamily. After the discovery of the enzyme in 1986, its cloning in 1987, and its first purification to homogeneity in 1990, several groups have focused their interest on this PKC isoenzyme and have reported on its expression, structural and enzymatic properties, and cellular functions. Some information has accumulated on the mode and structural requirements of activation and down-regulation of PKCδ. Recently, the role of phosphorylation of PKCδ, i.e. either autophosphorylation or phosphorylation by an exogenous protein kinase, for the regulation of its enzymatic activity could to some extent be elucidated and compared to that of other PKC isoforms. However, many questions remain to be answered. The same holds true for PKCδ-specific substrate phosphorylation and biological functions. Various more or less useful methods have been applied to elucidate PKCδ-specific functions in a given cell. Data accumulated that indicate a role of PKCδ in growth inhibition, differentiation, apoptosis, and tumor suppression. However, the knowledge on PKCδ-specific substrate phosphorylation resulting in these specific cellular effects is as yet extremely poor.

The PKC family

Protein kinase C (PKC) was initially identified and characterized as a proteolytically activated kinase called protein kinase M [1,2]. The proenzyme (PKC) was then shown to exhibit calcium- and phospholipid-dependent kinase activity independently of proteolytic activation [3]. In the presence of diacylglycerol (DAG), a well-known second messenger molecule [4,5], the calcium concentration necessary for stimulation of the enzyme was reduced to physiological levels [6]. DAG was known to be produced by phosphatidyl inositol turnover induced by cellular receptors upon interaction with numerous extracellular factors [4,5]. This indicated that PKC might play an important role in transmembrane signal transduction. Another key discovery was that PKC was the major intracellular receptor for the tumor-promoting phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [7,8]. When TPA was used as PKC activator *in vivo*, PKC proved to be linked to the signal-induced modulation of a wide variety of cellular processes, such as growth, differentiation, secretion, apoptosis, and tumor development. In 1986, the cDNAs of three PKC isoenzymes (α, β, γ) were cloned [9–11]. The PKCβ gene was found to generate two isoenzymes, βI and βII, by alternative splicing [12].

Since then, seven other isoenzymes [δ, ε, η, θ, ζ, λ(ι), μ] and PKC-related kinases (PRKs) have been detected (for reviews on PKC isoenzymes see [13–16]). The PKC family can be subdivided into three groups. The 'conventional' PKCs (cPKCs; α, $β_1$, $β_{II}$, γ) depend on calcium and are activated by diacylglycerol (DAG) or TPA; the 'novel' PKCs (nPKCs; δ, ε, η, θ) are also activated by DAG or TPA but are calcium-independent; the 'atypical' PKCs [aPKCs; ζ, λ(ι)] are calcium-independent and do not respond to DAG or TPA. The difference between the three subfamilies is reflected by differences in the molecular structures. The cysteine-rich sequences of cPKCs and nPKCs have been shown to be the site of interaction with phospholipid and diglyceride/phorbol ester activators [17]. The aPKCs lack one cysteine-rich sequence and are not activated by diglycerides or phorbol esters. However, a phorbol ester-responsive aPKCζ can be generated by fusion with the regulatory domain of PKCδ [18]. nPKCs lack the C2 region that determines Ca^{2+}-binding by the cPKC subfamily. PKCμ, is a novel PKC but contains a pleckstrin homology domain and a N-terminal hydrophobic domain and it lacks a region resembling the inhibitory pseudosubstrate regions of other PKC family members. Protein kinase C isoenzymes are activated by a very large number of extracellular signals and in turn modify the activities of a wide variety of cellular proteins including receptors, enzymes, cytoskeletal proteins, transcription factors, etc. Thus, PKC has a central position in cellular signal processing.

Cell-specific expression and subcellular localization of individual PKC isoenzymes indicate important isoenzyme-specific functions. To elucidate such functions, it will be necessary to study *in vitro* and/or *in vivo* the individual features of each isoenzyme, such as expression, post-translational modification, substrate specificity, subcellular localization and

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Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Cys, cysteine-rich motif; eEF-1α, eucaryotic elongation factor-1α; HSP, heat shock protein; RACK, receptor for activated C-kinase; AKAP, anchoring protein for cAMP-dependent protein kinase.

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signaling cross-talk with other proteins. In addition, the involvement of a PKC isoenzyme in a signaling pathway resulting in a specific cellular response can be investigated by several distinct methods such as overexpression of the enzyme or inhibition of enzyme expression or activity. The purpose of this article is to review our present knowledge regarding the PKCδ, a member of the nPKC subfamily, that attracted the interest of an increasing number of research groups over the last years.

Structure of PKCδ

In 1987, i.e. 1 year after cloning of the cPKCs (α, β, γ), a cDNA of PKCδ was cloned from a rat brain cDNA library by Ono *et al.* [19]. The PKCδ gene could be assigned to human chromosome 3 and mouse chromosome 14 [20]. The amino acid sequence of PKCδ showed 58% homology to PKCα [21]. As a member of the nPKC subfamily, PKCδ contains a carboxy-terminal catalytic domain with two conserved regions, C3 and C4, essential for catalytic activity and substrate binding, and an amino-terminal regulatory domain with an inhibitory pseudo-substrate sequence and two cysteine-rich Zn-finger-like sequences in the C1 region but lacking the C2 region. The cysteine-rich motifs of PKCδ are functionally not equivalent. Glutathione-S-transferase fusion proteins containing the second cysteine-rich motif (Cys2) of PKCδ bind the phorbol ester PDBu with high affinity, whereas no significant binding is seen for the Cys1 fusion protein [22]. Mutational studies indicate that Cys2 plays a key role in the translocation of cytosolic PKCδ to cellular membranes in response to an activation by phorbol ester [23]. Site-directed mutagenesis [24] and X-ray crystallographic studies [25] provided detailed understanding of the interaction between phorbol ester and Cys2 of PKCδ. Five of six cysteine residues as well as the two histidine residues involved in Zn^{2+} coordination were found to be critical for phorbol ester binding. In addition, mutations in other positions of Cys2 drastically reduce the interaction with the ligand. In a groove at the tip of Cys2 the oxygens at the C-3, C-4, and C-20 position of the phorbol moiety are thought to form hydrogen bonds with main-chain groups the orientation of which is controlled by a set of highly conserved residues. Phorbol ester binding caps the groove and forms a contiguous hydrophobic surface, explaining how the activator promotes insertion of PKCδ into membranes.

Purification and characterization of PKCδ

PKCδ was the first nPKC isoenzyme to be isolated from a tissue. In 1986, we reported on the isolation of a phorbol ester/phospholipid-activated, Ca^{2+}-unresponsive PKC-like enzyme [26–28], which later on was identified as PKCδ. The native enzyme was purified to homogeneity from porcine spleen and characterized [29]. It bound the phorbol ester TPA with high affinity (K_d = 9.6 nM) and could be activated *in vitro* for substrate and autophosphorylation by TPA (or diacylglycerol) plus phosphatidyl serine, but unlike the cPKCs (α, β, γ), not by calcium plus phosphatidyl serine. Activation was brought about also with various unsaturated fatty acids, particularly arachidonic acid. The affinity of native PKCδ for cofactors and substrates differed to some extent from that of conventional PKCs. In the meantime, both native and recombinant PKCδ have been partially or completely purified also from various other sources, such as rat brain [30], mouse hemopoietic cells [31], COS1 cells transfected with cDNA coding for rat [32] or mouse [33] PKCδ, and insect cells infected with recombinant baculovirus containing the cDNA of human PKCδ [34] as well as bacteria transfected with rat PKCδ cDNA [35]. The properties of the various PKCδ preparations did not differ significantly.

PKCδ has been found in most tissues and cell types [36,37]. The expression of the enzyme is ontogenetically regulated. Thus, the concentration of PKCδ in the epidermis and brain of newborn mice increases dramatically between day 7 and 14 after birth [36]. A detailed immunohistochemical investigation of the expression of PKCδ and other PKC isoforms in rat cerebellum was carried out by Chen and Hillman [38,39]. They observed a transient occurence of PKCδ in glia and later its appearance in selective groups of neurons. The expression of PKCδ was associated with specific activities of these cells.

Like other isoenzymes of the cPKC and nPKC groups, PKCδ is activated *in vivo* by DAG that is formed from phospholipids upon receptor-mediated activation of phospholipases [40]. While numerous signaling molecules, such as growth factors, hormones and cytokines, are known to cause DAG release and activation of PKC, reports on a selective activation of individual PKC isoenzymes are rare. The nPKCs δ and ε, but not the cPKCα, were found to be activated upon mitogenic stimulation of quiescent rat 3Y1 fibroblasts [41]. A selective activation of PKCδ may play a role in nerve growth factor-induced neurite outgrowth [42]. Cis-unsaturated fatty acids activate PKC *in vitro* and in intact platelets. According to Khan *et al.* [43], oleate activates mainly PKCδ. In none of these cases the mechanism of the apparently selective activation of PKCδ is known yet.

Similarly to other PKC isoforms, PKCδ is proteolytically degraded following activation by TPA *in vivo*. Physiological agonists causing DAG release, such as bombesin and PDGF, and DAG itself, are also able to cause degradation of PKCδ [40]. This so-called down-regulation occurs via the ubiquitin-proteasome pathway [44]. TPA treatment of mouse skin causes down-regulation of epidermal PKCδ with a half-life of around 8 h, followed by complete recovery within 72 h [36]. According to a report by Shih and Floyd-Smith [45], TPA induces down-regulation of PKCδ in the mouse B lymphoma cell line A20 not only at the protein but also at the mRNA level. Down-regulation of PKCδ in Swiss 3T3 cells appears to be cell-cycle-dependent, operating only in the G0/G1 [46]. Paradoxically, the PKC activator bryostatin 1 protects PKCδ, but not other PKC isoforms, against TPA-induced down-regulation in mouse keratinocytes [47]. Protection is observed at 100 nM to 1 μM, whereas at concentrations of less than 1 nM, bryostatin 1 itself causes down-regulation of PKCδ to a similar extent as does TPA. Studies with reciprocal chimeras constructed by exchanging the regulatory and catalytic domains of PKCα and PKCδ grants that the catalytic domain of PKCδ confers protection by bryostatin 1 against down-regulation and contains the isotype-specific determinants involved in the unique effect of bryostatin 1 on PKCδ [48].

Recently, various PKC-specific inhibitors have been identified or developed (for a review see [49]). Among these, rottlerin was found to possess some specificity for PKCδ [50]. However, no absolutely PKCδ-specific inhibitor is available as yet. The staurosporine-related compound K252a and the staurosporine-derived compound Gö 6976 are able to differentiate between subgroups of PKC [28,51,52] in that these inhibit Ca^{2+}-responsive isoforms much more effectively than Ca^{2+}-unresponsive isoforms including PKCδ.

Expression of PKCδ

Contrary to PKCβ and γ, the PKCδ gene has not been cloned yet. Thus, the regulatory regions of the gene and respective factors that might affect the onset of transcription are not

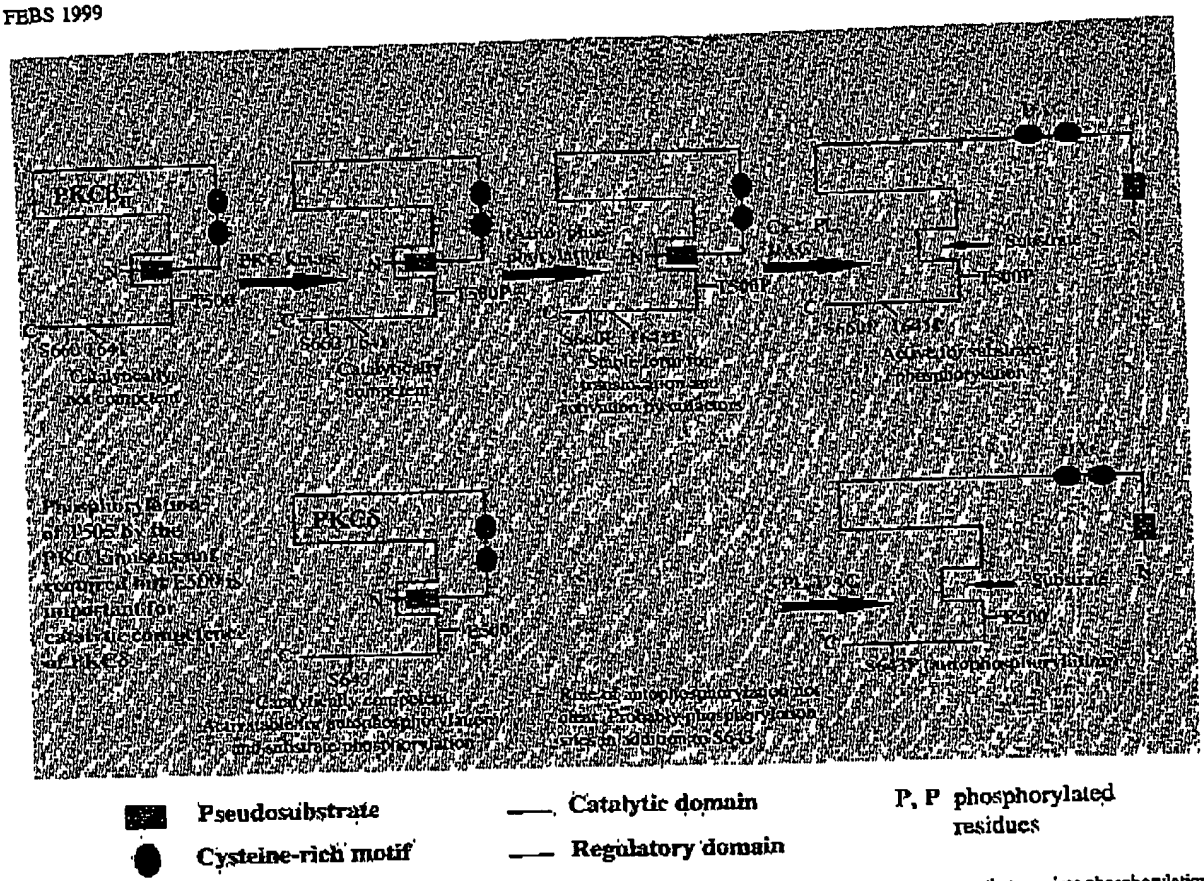

Fig. 1. Phosphorylation and activation of PKCβII and PKCδ. PKCβII is synthesized as a catalytically incompetent enzyme that requires phosphorylation by the PKC kinase (PDK1) of threonine 500 (T500) in the activation loop for catalytic competence. Subsequent (auto)phosphorylation of threonine 641 (T641) and serine 660 (S660) is necessary for maintaining a catalytically competent conformation and the regulation of subcellular distribution [69]. It is not yet absolutely clear whether both sites (T641 and S660) are autophosphorylated or another kinase is involved. The stable catalytically-competent form of PKCβII can be activated by Ca^{2+}, phospholipid (PL) and dicylglycerol (DAG) for substrate phosphorylation. PKCδ is synthesized as a catalytically competent enzyme that is activatable by PL and DAG for autophosphorylation and substrate phosphorylation. Phosphorylation of T505 (corresponding to T500 of PKCβII) is not required for catalytic competence [35]. The negative charge in the activation loop that is necessary for catalytic competence of PKCδ is probably provided by glutamic acid 500 (E500) [67]. PKCδ is autophosphorylated *in vitro* on serine 643 (S643; [67]). Probably, additional (auto)phosphorylation sites exist, but have not been identified. The role of PKCδ (auto)phosphorylation is not yet clear.

known. However, up-and down-regulation of the expression of PKCδ in various cells and tissues was reported and evidence for an isotype-specific expression was obtained. Thus, estrogen treatment increases the level of PKCδ protein in rabbit [53] and rat copora lutea [54], probably by a post-transcriptional mechanism, whereas the levels of other PKC isoforms remain relatively constant. Interleukin-1β induces the expression of PKCδ and ε, but not of α and ζ, in NIH 3T3 cells [55]. 1,25-Dihydroxyvitamin D_3 stimulates the expression of PKCδ and α in acute promyeolocytic NB4 cells via a nongenomic mechanism [56]. The authors propose that by increasing the expression of these PKC isoforms the cells are primed for TPA-induced monocyte differentiation. Assert *et al.* [57] reported on a 400% increase of PKCδ-mRNA in human T84 cells 24 h after TPA treatment. However, the level of PKCδ protein was not elevated to the same degree as that of mRNA. Elevated PKCδ- (and ε) mRNA levels were reported to occur also upon treatment of αT3–1 cells with gonadotropin-releasing hormone. This process appeared to be autoregulated by PKC [58]. Cross-talk between PKC isoenzymes also might regulate PKC levels. Overexpression of PKCα in Baf3 and 32D cells leads to an elevation of the endogenous PKCδ protein level, altering its mRNA transcription and degradation [59]. Stable transfection of the human keratinocyte line HaCaT with mutated cellular Ha-ras results in a selective loss of PKCδ-mRNA and protein that is mediated by TGFα [60]. This is in accordance with a proposed tumor-suppressive role of PKCδ.

Phosphorylation of PKCδ

Phosphorylation in the activation loop by a 'PKC kinase'? PKCδ seems to differ from other PKC isoenzymes in its mechanism of post-translational regulation. PKCα [61] and $β_{II}$ [62] are known to require phosphorylation of threonine 497 and 500, respectively, in the so-called activation loop for enzymatic activity. Very recently, the 'PKC kinase' responsible for this phosphorylation has been found to be the phosphoinositide-dependent kinase PDK1 [63–65]. This kinase is most likely not

present in bacteria. This would explain that PKCα could not be expressed as an active recombinant enzyme in bacteria [66]. Gschwendt and coworkers showed that PKCδ, contrary to PKCα, can be expressed in a catalytically competent form in bacteria [35]. Recombinant PKCδ partially purified from bacteria had a specific activity comparable to that of native PKCδ from porcine spleen. In addition, it was demonstrated by site-directed mutagenesis that phosphorylation of threonine 505 in PKCδ, unlike that of the corresponding threonines in PKCα and β_{II}, is not essential for a permissive activation of PKCδ (see Fig. 1). The negative charge in the activation loop of PKCδ that is necessary for catalytic competence of the kinase is probably provided by glutamic acid 500. Mutation of this acidic amino acid residue to valine caused an 80% loss of kinase activity ([67]; see Fig. 1). These results do not exclude modulation of PKCδ activity due to phosphorylation by another kinase, but they indicate that PKCδ and other PKC isoenzymes are differentially regulated by post-translational modification.

Autophosphorylation. Like many other protein kinases, PKC isoenzymes undergo autophosphorylation the functional role of which is still a matter of debate. PKCδ purified to homogeneity from porcine spleen exhibits a particularly high potential for autophosphorylation *in vitro*, which is around four times that of the cPKCs α, β, γ. The stoichometry and phosphoamino acid analysis of autophosphorylation *in vitro* suggested that fully phosphorylated PKCδ contains two phosphoserine residues and one phosphothreonine [29]. *In vitro* PKCδ accepts not only ATP but also GTP as phosphate donor for autophosphorylation [68].

While $PKC\beta_{II}$ was found to be autophosphorylated *in vivo* on threonine 641 and serine 660 [69,70], the *in-vivo* (auto)phosphorylation sites of PKCδ have not been unequivocally identified yet. Recently, site-directed mutagenesis of serine 643 and comparison of *in-vivo* phosphorylated wild-type and alanine mutant by phosphopeptide mapping indicated that serine 643 is an *in-vivo* phosphorylation site of PKCδ [71]. Phosphorylation of this site appeared to be important for enzymatic activity. These data did not indicate whether serine 643 is autophosphorylated or phosphorylated by another protein kinase. Stempka *et al.* [67] identified serine 643 as an *in-vitro* autophosphorylation site of PKCδ by MALDI mass spectrometry of tryptic peptides derived from recombinant PKCδ that was purified from bacteria or baculovirus-infected insect cells. In contrast to the report by Li *et al.* [71], Stempka *et al.* [67] were unable to demonstrate any loss of kinase activity upon serine 643 to alanine mutation. Thus, the role of PKCδ autophosphorylation is not yet clear, particularly, as probably additional, as yet unidentified (auto)phosphorylation sites exist (see Fig. 1).

Phosphorylation of tyrosine residues. PKCδ was the first PKC isoenzyme found to be functionally modulated by tyrosine phosphorylation. The tyrosine kinase Src phosphorylates PKCδ *in vitro* [72]. *In-vitro* tyrosine phosphorylation occurs only in the presence of an activator, i.e. TPA, and thus appears to be restricted to the activated form of the enzyme. Possibly, the phosphorylation sites are exposed only upon an activator-induced conformational change of PKCδ. Tyrosine phosphorylation increases the apparent molecular mass of PKCδ and modifies its activity towards some substrates. Tyrosine phosphorylation of PKCδ was demonstrated also *in vivo* in response to treatment of cells with various agents, such as TPA, PDGF, TGFα, carbachol, substance P, ligand of IgE receptor, extracellular ATP or UTP, and H_2O_2 [73–79], and in response to transfection of murine keratinocytes with oncogenic Ha-ras [80].

Upon treatment of PKCδ-overexpressing cells with PDGF or TPA, tyrosine-phosphorylated PKCδ was found to be localized in the membrane fraction and its activity increased [74]. According to Konishi *et al.* [78] and Kadotani *et al.* [81], tyrosine phosphorylation of PKCδ *in vivo* creates a modified enzyme that is active without or at least with a lower concentration of lipid cofactors. Upon treatment of cells with H_2O_2, not only PKCδ but also PKC α, β_{II}, γ, ε, and ζ were found to be tyrosine-phosphorylated and activated [78]. In contrast to these studies, tyrosine phosphorylation was reported to diminish the activity of PKCδ [75,77,80]. According to *in vitro* studies [72], it seems to depend on the substrate whether PKC activity is elevated or reduced by tyrosine phosphorylation. In other words, tyrosine phosphorylation might regulate the specificity of the kinase toward a given substrate. For example, PKCδ that was phosphorylated on tyrosine upon activation of the high-affinity receptor for IgE has diminished activity toward the γ-chain peptide of this receptor as a substrate but not toward histones or myelin basic protein peptide [77]. It is not known yet whether PKCδ is phosphorylated *in vivo*, as *in vitro*, by Src or another tyrosine kinase. However, it was shown that PKCδ associates with v-Src and is phosphorylated on tyrosine and activated in v-Src-transformed fibroblasts [82,83]. Very recently, association with Src and tyrosine phosphorylation of PKCδ was observed also in MCF-7 human breast cancer cells in the absence of overexpression of either PKCδ or Src [84]. Moreover, various mitogenic agents, including the phorbol ester TPA, activate Src-family kinases in Swiss 3T3 cells [85], epidermal keratinocytes [75], or MCF-7 cells [84]. Even though activation of the EGF receptor results in tyrosine phosphorylation of PKCδ, the EGF receptor tyrosine kinase is not able to phosphorylate PKCδ *in vitro* [72]. Thus, it is very well conceivable that the EGF receptor signal is mediated by Src. Recently, the c-Abl tyrosine kinase was found to be associated with PKCδ in diverse cell types, such as MCF-7 cells, U-937 cells, and HL-60 myeloid leukemia cells [86]. Treatment of those cells with DNA-damaging agents is associated with c-Abl-dependent phosphorylation of PKCδ and translocation of PKCδ to the nucleus. c-Abl phosphorylates and activates PKCδ also *in vitro*. These data suggest that, possibly in addition to Src, Abl is a PKCδ tyrosine kinase *in vivo*.

Site-directed mutagenesis indicated that tyrosine 52 [87], tyrosine 187 [88], tyrosine 512 and 523 [78] are all sites for tyrosine phosphorylation of PKCδ *in vivo*. However, these sites have not been identified unequivocally yet, for example by MALDI mass spectrometry of tryptic peptides of tyrosine-phosphorylated PKCδ.

Biological functions of PKCδ

Various methods have been applied to elucidate PKCδ functions. Differential down-regulation of PKC isoenzymes by TPA, bryostatin, etc., can be used to selectively remove from or retain PKCδ in a cell. A TPA-induced physiological response in such cells or its lack is then attributed to the action of PKCδ. This method was applied to show that PKCδ is involved in the stimulation of the Na^+–H^+ exchanger in C_6 glioma cells [89], in phosphoinositide hydrolysis and PGE_2 formation [90], and in keratinocyte differentiation [47]. Similarly, it was demonstrated that selective activation of PKCδ might play a role in neuritogenic signals in PC12 cells [42]. Even if selectivity and duration of down-regulation have been carefully checked, this method is of limited use only. The effects of TPA are manifold and therefore, one cannot exclude the possibility that the observed cellular response or its lack is the consequence of a TPA effect other than down-regulation of PKC. Alternatively, PKCδ can be inhibited by expression of a dominant negative

ATP-binding mutant of the enzyme [91] or its expression can be suppressed by treatment of cells with antisense oligonucleotides to PKCδ. These methods are much more specific than TPA-induced down-regulation. The use of a dominant negative mutant indicated that PKCδ is involved in Sis-induced transformation of NIH 3T3 cells via the activation of genes containing the TPA responsive element, but not via the Ras cascade [92]. Antisense oligonucleotides were used to demonstrate a role of PKCδ in differentiation of murine erythroleukaemia cells [93] and in the α_1-adrenergic activation of Na–K–2Cl cotransport [94].

A method frequently used for studying PKCδ function is the establishment of stable cell clones that overexpress PKCδ upon transfection with corresponding expression vectors. Changes in cellular processes, observed with and/or without treatment of the transfected cells with TPA, are then regarded to be mediated by PKCδ. However, overexpression is not necessarily physiologically relevant. For example, the overexpressed PKC isoenzyme may overwhelm the endogenous enzyme and thus cause nonspecific localization and substrate phosphorylation. Several groups reported that overexpression of PKCδ causes growth inhibition, as for instance in CHO cells [95], in smooth muscle cells [96], in NIH 3T3 fibroblasts [97], in human glioma cells [98], and in capillary endothelial cells [99]. In these studies, additional effects of PKCδ overexpression were reported, such as changes in cell morphology, decreased cell density, and suppression of G_1 cyclin expression. In some reports changes were accentuated by treatment of cells with the phorbol ester TPA. Overexpression of PKCδ (and α), but not β_I, ε, η, or ζ, enables the murine myeloid 32D cells to differentiate into mature macrophages after treatment with TPA [100] or PDGF [101]. Using overexpression of reciprocal δ and ε chimeras, it was shown that the catalytic domain of PKCδ mediates the TPA-induced differentiation of 32D cells [102]. Overexpression of PKCδ in NIH 3T3 cells increases the sodium-dependent phosphate uptake [103]. By application of this method it was shown also that PKCδ, but not β_1, ε, or ζ, inhibits the activation of the STAT signaling pathway by the Tec-family kinase Bmx [104]. Moreover, it was demonstrated that PKCδ (and PKCα) mediates the transcription of TPA-inducible genes via both AP-1 and non-AP-1 sequences [105]. Ras-dependent signal transduction is involved in the activation of AP-1 by PKCδ [106]. Contrary to that, PKCδ activates the MEK-ERK pathway independently of Ras and dependently of Raf, as was demonstrated by expression of a constitutively active mutant of PKCδ [107]. Alternatively to transfection of cells with a PKCδ cDNA and overexpression of PKCδ, the enzyme can be directly introduced into permeabilized cells. By applying this method it was demonstrated that PKCδ is involved in exocytosis [108].

Besides the involvement of PKCδ in the regulation of cell growth and differentiation, this PKC isoform might play a role also in apoptosis and tumor development. Emoto *et al.* [109–111] demonstrated that apoptosis induced in human myeloid leukemia cells by TNFα, ara-C, anti-Fas-antibody, radiation, etc. was associated with proteolytic activation of PKCδ by an ICE-like protease. It was shown that the cysteine protease CPP32 is responsible for this cleavage. The proteolytic generation of a 40-kDa catalytic kinase fragment (aa 331–676) upon induction of apoptosis can be suppressed by overexpression of the anti-apoptotic proteins Bcl-2 and Bcl-x_L. Overexpression of the catalytic kinase fragment of PKCδ, but not of full-length PKCδ or a kinase-inactive fragment, caused phenotypic changes associated with apoptosis. Similar results were reported by Mizuno *et al.* [112]. However, the authors demonstrated apoptosis-associated limited proteolytic cleavage not only of PKCδ but also of other nPKC isotypes, such as PKC ε and θ, whereas PKCα, β, and ζ were not affected. Ceramide-induced cytosolic translocation of PKCδ and ε was observed in HL60 cells and may be a prerequisite for the proteolytic activation of the kinases in apoptosis [113]. Very recently, Bharti *et al.* [114] reported on an interaction of the catalytic fragment of PKCδ with and phosphorylation of the DNA-dependent protein kinase (DNA-PK) resulting in the inactivation of this kinase. DNA-PK is essential in the repair of DNA double-strand breaks. Thus, interaction of PKCδ and DNA-PK may contribute to DNA damage-induced apoptosis.

The possible role of PKCδ in the induction of apoptosis may be at least one reason for its tumor-suppressive action that has been indicated by several reports. Overexpression of PKCδ in the liver particulate fraction of low protein-fed rats correlates with inhibition of the development of preneoplastic lesions in diethylnitrosamine-induced rat hepatocarcinogenesis [115]. Expression of PKCδ in human keratinocytes is significantly reduced upon ras transformation [60]. Induction of a transformed phenotype by TPA in c-Src overexpressing rat fibroblasts appears to be due to depletion, and not activation, of PKCδ [116], since it could be evoked by expression of a dominant negative PKCδ mutant as well as treatment with the PKCδ inhibitor rottlerin. Moreover, bryostatin 1, which inhibits TPA-induced down-regulation of PKCδ specifically [47], blocks TPA-induced tumor promotion in mouse skin [117]. TPA-induced tyrosine phosphorylation of PKCδ, for instance by Src, might be a signal for its proteolytic degradation (Gschwendt *et al.*, unpublished results) eliminating protection against tumor development. Indeed, v-Src was shown to be associated with and down-regulate PKCδ [83]. Very recently, Reddig *et al.* [118] demonstrated that transgenic mice overexpressing PKCδ in the epidermis showed a dramatic reduction in the formation of skin tumors upon treatment with 7,12-dimethylbenz[*a*]anthracene/TPA.

Substrates and binding proteins

One of the most important tasks in learning to understand the function of individual PKC isoenzymes is the search for physiological substrates and associating proteins. A large number of proteins are known that are phosphorylated by PKC. However, the knowledge of proteins that are selectively phosphorylated by a distinct PKC isoform is extremely scarce. Essentially two mechanisms are conceivable that may enable a PKC isoenzyme, like PKCδ, to phosphorylate a substrate protein selectively and thus transduce an isoenzyme-specific signal. A PKC isoenzyme might recognize a consensus sequence of a substrate protein that is not or just poorly recognized by the other isoenzymes. Alternatively or in addition, colocalization of a PKC isoenzyme with substrates in a cell compartment might determine the specificity of substrate phosphorylation.

An example for a substrate protein with a specific recognition site for PKCδ is the eucaryotic elongation factor-1α (eEF-1α), which is phosphorylated at threonine 431 selectively by PKCδ [119]. Moreover, the eEF-1α peptide 422–443 containing the phosphorylation site is an absolutely specific substrate for the δ-type of PKC. A single basic amino acid close to the phosphorylation site is essential for specific recognition and phosphorylation of the peptide by PKCδ. Substitution of this basic amino acid by alanine abolishes the ability of PKCδ to phosphorylate the peptide, and insertion of additional basic amino acids in the vicinity of the phosphorylation site causes a complete loss of selectivity. Phosphorylation and activation of

eEF-1 in intact cells upon treatment with TPA was reported previously [120,121].

As another PKCδ-specific substrate the γ-chain of the high-affinity receptor for immunoglobulin E that is associated with PKCδ has been described [122]. The γ-chain is phosphorylated *in vivo* on a threonine residue in response to PKC activation. The phosphorylation correlates with the endocytosis of the receptor. However, the phosphorylation site has not been identified and the essential amino acid(s) for specific recognition by PKCδ are not known yet. Jideama *et al.* [123] reported on a PKCδ-specific phosphorylation site in troponin I. PKCδ was found to be unique among all PKC isoenzymes in its ability to mimic protein kinase A in phosphorylating Ser 23/Ser 24 of this protein and in reducing Ca^{2+} sensitivity of Ca^{2+}-stimulated MgATPase of reconstituted actomyosin S-1. In addition PKCδ, like PKCα, phosphorylates Ser-43/Ser-45 and reduces maximal activity of MgATPase. Most of the *in-vitro* phosphorylation sites were confirmed *in situ* in adult rat cardiomyocytes. The heat-shock protein-25/27 (HSP-25/27) was shown to be efficiently phosphorylated by PKCδ and to a lesser extent by PKCα, whereas other PKC isoforms (β_1, β_2, ε, ζ) were not effective HSP kinases [124]. A physiological significance of this phosphorylation was indicated by the finding that HSP-27 was more strongly phosyphorylated *in vivo* in rat corpora lutea of late pregnancy, a developmental stage in which PKCδ is abundant and active. Moreover, late-pregnancy luteal extracts contain a lipid-sensitive HSP-kinase activity which is copurified with PKCδ.

Isoenzyme-specific binding proteins and scaffolding or anchoring proteins, such as RACKs, AKAPs, etc. [125], which are able to bind the isoenzyme and the substrates, most likely play an essential role in colocalizing kinase and substrate.

Little is known about the localization of PKCδ in or translocation to cell compartments that might determine selectivity of substrate phosphorylation. Taken together, data are accumulating that indicate an association of PKCδ with nuclear and cytoskeletal structures of the cell. In most cases, however, a link to selective phosphorylation of substrate proteins by PKCδ is missing. Leibersperger *et al.* [36] reported on a nuclear or perinuclear localization of PKCδ in skin sections. Similar results have been obtained for bovine brain cells [126]. In cultured cells of neuronal origin, PKCδ was found in nucleoli [127]. A redistribution of PKCδ from a peri-nuclear/cytoplasmic compartment to a putatively cytoskeletal location upon activation of a human T cell line was reported by Keenan *et al.* [128]. A TPA-induced association of PKCδ with the cytoskeleton was also found in beta cells. Oleic acid inhibits translocation to the cytoskeleton and rather induces an association with membranes, indicating that different activators of PKCδ may have different effects on the localization of the enzyme within the cell [129]. In HL60 cells, PKCδ was found to colocalize with the PKC substrate vimentin in the cytosol and perinuclear region [130]. Barry & Critchley [131] demonstrated that in Swiss 3T3 cells serum induced the recruitment of PKCδ together with focal adhesion kinase (pp125FAK) to newly formed focal adhesions.

Almost nothing is known about proteins that bind specifically PKCδ and might be important for intracellular targeting of this PKC isoform. Receptors for activated C-kinases (RACKs) specific for $PKC\beta_{II}$ and PKCε were described. Moreover, the existence of receptors for inactive C-kinase (RICKs), with characteristics similar to the anchoring proteins for cAMP-dependent protein kinase (AKAPs), has been discussed (for a review see [125]). PKCδ-specific RACKs or RICKs are not known yet. However, data reported by Jaken and coworkers indicate that the regulatory domain of PKCδ and other PKC isoenzymes is involved in the interaction with other proteins [132,133]. Indeed, a binding site of PKCδ for a putatively specific RACK was found to be localized in the N-terminal V1 domain (amino acids 1–142). This domain resembles to some extent the C2 domain of cPKCs, which is known to interact with RACKs. A peptide corresponding to this PKCδ domain selectively prevented translocation of PKCδ upon cell stimulation [134]. Similarly, binding of the PKC substrate GAP-43 to this domain of PKCδ was reported [135]. Some other proteins, such as MARCKS [136] and SRBC [137] are not only phosphorylated by PKCδ and other PKC isoenzymes but also bind to the regulatory domain of PKCδ with a rather high affinity (K_m = 20–60 nM). Therefore, a PKC receptor function of these proteins has been discussed. An isoenzyme-specific interaction of PKCδ with phosphatidylinositol 3-kinase was detected by coimmunoprecipitation [138]. The association is modulated by cell activation. Its function is not yet known.

Conclusion

Within the last decade, after the discovery of PKCδ in 1986 and its cloning in 1987, quite a number of data have accumulated. The native enzyme as well as recombinant enzymes were purified and characterized. Particularly the role of the second cysteine-rich motif in binding the activating phorbol esters was studied in detail. Beside the activation by cofactors, that is distinct with cPKCs, nPKCs, and aPKCs, most properties of PKCδ known so far do not differ significantly from those of all other PKC isoenzymes. The selective protection by bryostatin 1 from TPA-induced down-regulation, the lacking requirement for threonine phosphorylation in the activation loop for permissive activation of the enzyme, and possibly tyrosine phosphorylation as a response to extracellular stimuli were all found to be special characteristics of PKCδ. Tyrosine phosphorylation as a response to extracellular stimuli, however, might occur also with other PKC isoforms, as indicated by a recent report. Several studies suggested PKCδ to be involved in the regulation of cell growth and differentiation and to play a role in apoptosis and tumor development. However, the signaling pathways leading to these cellular processes, particularly the role of PKCδ in this signaling, is not or just poorly understood. A major drawback is the missing knowledge of physiological substrates and PKCδ associated proteins determining the specific functions of this PKC isoenzyme in a cell. Therefore, one of the most important tasks in future studies on PKCδ will be the search for proteins, including substrate proteins, that specifically interact with PKCδ. Presently, methods like the yeast two-hybrid system or overlay techniques are applied for this purpose.

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